Age-Dependent Vulnerability of the Striatal Mitochondrial to 3-Nitropropionic Acid

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Abstract. From studies to date, there is considerable evidence suggesting that energy impairment may be a common biochemical mechanism underlying the etiology of a number of metabolic disorders. Metabolic disorders are the result of an abnormal metabolic function at the cellular level, which can be inherited due to a genetic mutation, or acquired as a result of a series of multigenic diseases such as diabetes. The impact of metabolic disorders such as diabetes is growing in the state of Ohio. The statistics from the Diabetes Association of Greater Cleveland show a very high rate of diabetic complications in Ohio, where over 800,000 people have diabetes- about 1/16 of the population in Ohio. To study the effect of metabolic disorder at a cellular level, we have utilized 3-nitropropionic acid (3NP) to induce energy impairment and assess the metabolic response of two age groups to such stress. 3NP has long been known to inhibit succinate dehydrogenase activity of the Krebs cycle and the respiratory chain in mitochondria. Systemic administration of 3NP results in a selective striatal lesion in rodent and primate models. 3NP-induced toxicity has previously been demonstrated to be age-dependent, in which the older animals are more vulnerable to 3NP toxicity than the younger animals. The present study was designed to investigate whether 3NP treatment demonstrates variable potency in the isolated mitochondria from the striatum of younger (two-month-old) versus older (10-month-old) Sprague-Dawley rats.

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INTRODUCTION

Oxidative phosphorylation catalyzes the oxidation of glucose and other substrates by oxygen with a concomitant energy transduction into ATP. One of the major enzymes in oxidative phosphorylation is succinate dehydrogenase (SDH). SDH is located on the matrix side of the inner mitochondrial membrane and consists of four nuclear-encoded protein subunits (Ackrell, Kearney et al. 1975; Ackrell, Cochran et al. 1989). SDH catalyzes the oxidation of succinate to fumarate by reducing the flavine adenine dinucleotide (FAD) on its flavoprotein subunit. The unique position of SDH on the matrix side of the inner mitochondrial membrane enables SDH to participate in both the Krebs cycle and the electron transport chain. This property of SDH suggests a pivotal role for this enzyme in aerobic respiration. 3NP is an irreversible inhibitor of SDH. The inactivation of SDH by 3NP is a two step process in which 3NP is first oxidized to 3-nitroacrylate by a two-electron transfer to the FAD subunit of SDH, followed by the interaction of 3-nitroacrylate with the thiol group of SDH forming a thioether which irreversibly inactivates the SDH activity (Fig. 1) (Coles, Edmondson et al. 1979).

Previous reports have indicated an age-dependent susceptibility to 3NP toxicity in the rat model in which the younger experimental animals were more resilient to 3NP toxicity than the older animals (Bossi, Simpson et al. 1993; Brouillet, Jenkins et al. 1993). However, 3NP administration results in significant reduction in SDH activity throughout the brain of both the young and the old animals, but the reasons for variable susceptibility between different age groups is not well understood.

A number of previous reports have examined the activity of succinate dehydrogenase in aging. These reports range from an increase in SDH activity due to an increase in SDH enzyme in rat heart (Velez, Machado et al. 1985) to no change in SDH activity in brain (Bowling, Mutisya et al. 1993), and significant decline in SDH activity in hypothalamus (Shemyakov 2001), lymphocytes (Drouet, Lauthier et al. 1999) and skeletal muscle (Boffoli, Scacco et al. 1994). An interesting observation reported by Baker and Santer indicated a significant decrease in maximal velocity (V_{max}) of SDH activity without any change in Michaelis constant (K_m) in the rat superior cervical and celiac-superior mesenteric ganglia, suggesting an overall decrease in maximal metabolic activity of SDH in aged sympathetic neurons (Baker and Santer 1990). A comprehensive evaluation of the above studies implies that vulnerability of SDH activity in aging differs in various organs, tissues and cell populations.

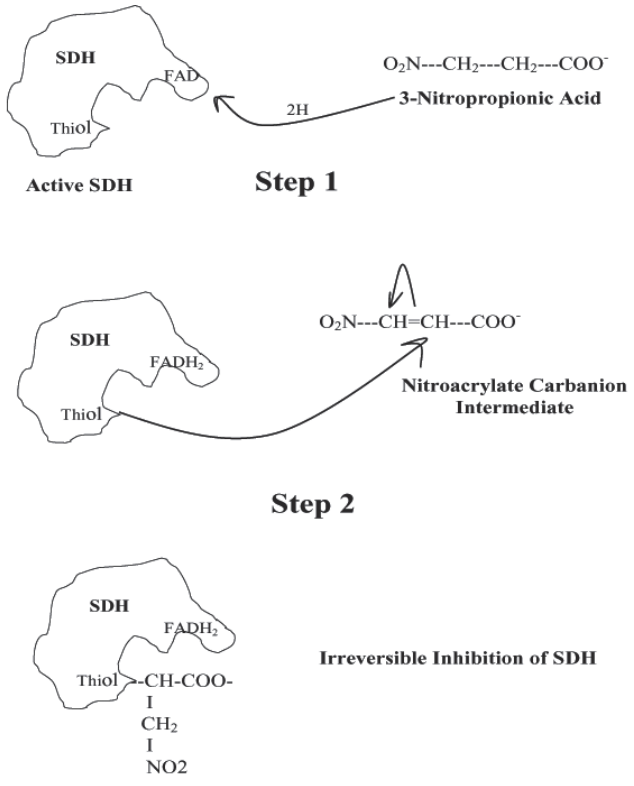

Figure 1. Succinate dehydrogenase inhibition by 3-nitropropionic acid. Succinate dehydrogenase inhibition is believed to be the result of a two-step process. First, the dianion form of 3NP is oxidized to 3-nitroacrylate. Second, 3-nitroacrylate interacts with thiol group of SDH, forming a thioether which irreversibly inhibits SDH activity.

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To better understand the age-dependent variability associated with 3NP toxicity, we examined the effects of 3NP on respiration of isolated mitochondria in two and ten month-old Sprague-Dawley rats. For comparative purposes, we examined the isolated mitochondria from cerebellum of the same animals since cerebellum is relatively spared in 3NP-induced toxicity. Sequential addition of 3NP resulted in similar decline in the rate of respiration between the striatum and cerebellum in the young animals, whereas in older animals, similar concentration of 3NP more efficiently inhibits the mitochondrial respiration in the striatum than that of the cerebellum. Following 3NP treatment, there was no increase in Reactive Oxygen Species (ROS) production in either the striatum or cerebellum of each age group. In contrast, the addition of Ca^{2+} (50-150 μM) resulted in a concentration-dependent increase in ROS generation in the older animals, while no significant changes were observed in younger animals. Moreover, in the older animals, the calcium insult resulted in a significantly higher ROS production in the striatum compared to the cerebellum of the same animal. The results of current study suggest that 3NP administration equally affects the mitochondrial respiration in the striatum and cerebellum of young animals; however, in the aged animals the striatal respiratory rate is more vulnerable to inhibition by 3NP than the cerebellum. The results of this study dispute the reactive oxygen species hypothesis of aging since there was no immediate increase in reactive oxygen species following 3NP treatment in either striatum or cerebellum, but the addition of calcium insult resulted in a concentration-dependent increase in reactive oxygen species in older animals.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Harlan Labs (Indianapolis, IN). At the time of experiments, the younger animals were two months old, while the older animals were ten months of age. Animals were housed individually and maintained on a 12h dark/light with food and water available *ad libitum*. All experimental protocols involving animals are in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Guidelines for the Use of Animals in Neuroscience Research.

Mitochondrial Preparation

Isolated mitochondria were prepared as previously described with slight modifications (Sullivan 1999). Briefly, two- and 10-month-old Sprague-Dawley rats (n=6 per group) were anesthetized by sodium pentobarbital (60 mg/kg), the brains were removed, and the striatum and cerebellum were carefully dissected. All the following steps in mitochondrial isolation were performed at 4°C. The dissected tissue was minced in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.1 % BSA, 1 mM EDTA, pH 7.2) and was rinsed with 10 ml homogenization buffer to remove residual blood. Next, the minced tissue was processed (eight strokes) using a hand-held tissue homogenizer (Thomas Scientific). The resulting homogenate was centrifuged for three minutes at 1300 x g, the supernatant was removed, centrifuged at 13,000 x g for 10 minutes and the resulting pellet was placed in nitrogen cell bomb and exposed to 1000 psi for five minutes to disrupt synaptosomal membranes. The pellets were resuspended in EGTA-free isolation buffer and centrifuged at 10,000 x g for 10 minutes. The mitochondrial respiration analysis and *2',7'-dichlorofluorescin diacetate* assay (DCF) were performed immediately after mitochondrial isolation. Mitochondrial protein concentration was determined using a Pierce BCA kit.

Reactive Oxygen Species Production

Reactive oxygen species (ROS) production was measured using the indicator DCF-DA, (Molecular Probes, Eugene, OR) as previously described (Sullivan 1999; Sullivan 2000). Briefly, 100-150 μg of isolated mitochondrial protein was incubated in a total volume of 200 μl respiration buffer (215 mM mannitol, 75 mM sucrose, 1% BSA, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES) at 37°C for 15 minutes in the presence of 10 μM DCF-DA, which was made fresh before each use. The relative amount of mitochondrial free radical generation in the presence of 3NP or calcium (50 μM-150 μM) was monitored by measuring changes in fluorescence resulting from DCF-DA oxidation product, DCF, using a CytoFluor 4000 fluorometric plate reader (excitation 490 nm, emission 526 nm). Addition of H_2O_2 was utilized as a positive control and increased DCF fluorescence in a linear style.

Mitochondrial Respiration

The respiratory activity of mitochondria from the striatum and cerebellum was measured within 30-60 minutes of their isolation. Succinate was used as a substrate to measure the respiratory activity associated with SDH activity. Mitochondrial respiration was assessed using standard polarography methods at 37°C with pyruvate and malate (5 mM and 2.5 mM) as oxidative substrates as previously described by Sullivan and colleagues (Sullivan, Dube et al. 2003). Mitochondrial uncoupling protein (UCP) mediated proton conductance was measured as increased fatty acid-induced respiration, which was then compared to maximum respiration induced by the chemical uncoupler carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP).

Statistical Analysis

In the 3NP titration experiments, 3NP-induced inhibition of mitochondrial respiratory activity was measured after each addition of 3NP (1mM per addition) and was expressed as a percentage of the maximum respiration measured in the presence of FCCP. The data for 3NP titration and DCF assay (t_1= 0 min vs. t_2=15 min) in each region was analyzed utilizing a 2-tailed paired t-test, while the comparison in the rate of respiration and ROS generation from different regions were performed by 2-tailed unpaired t-test. In each case, the t-test was followed by a Bonferroni/Dunn test for multiple comparisons. All values are expressed as the mean values ± the standard error of the mean of "n" observations, and a probability level of $p<0.05$ was considered significant.

RESULTS

3NP Titration

Approximately 30 seconds following the addition of FCCP, 2 μl 3NP (1 mM final concentration) was added every minute to the respiration reaction until the SDH activity was completely depleted. The results indicated a final concentration of 8 mM for 3NP to inhibit the respiratory activity of mitochondria in both the striatum and cerebellum of young rats (Fig. 2). The sequential introduction of similar concentration of 3NP in the older animals also resulted in inhibition of mitochondrial respiration in the striatum and cerebellum of aged animal. However, the respiratory

rate of the striatal mitochondria in the older animals was more effectively inhibited (Fig. 3).

Regional Respiration

To examine whether isolated mitochondria from the striatum and cerebellum of each age group demonstrate different rates of respiration, we evaluated the oxygen consumption of mitochondria in state III respiration. Although the mitochondria preparation from younger animals consistently demonstrated a higher oxygen consumption capacity, upon statistical analysis, there was no significant difference between the striatum and cerebellum of each group. Furthermore, the rate of oxygen consumption in the striatum of aged animals was steadily lower than that of the cerebellum, but there was no statistically significant difference in oxygen consumption between the two age groups (Table 1).

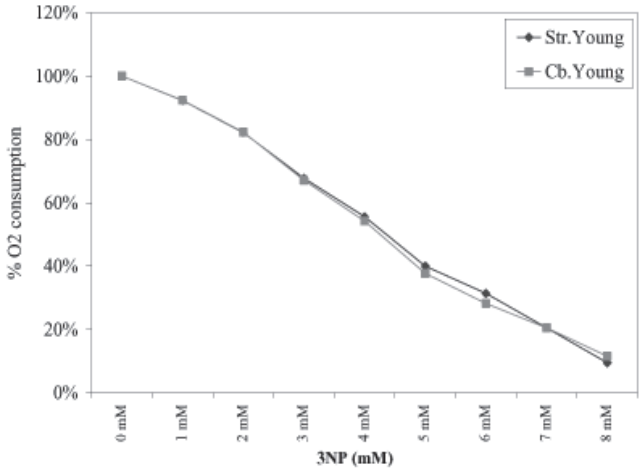

FIGURE 2. Mitochondrial respiration and 3NP treatment in young animals. Both the striatum and cerebellum of two-month old Sprague-Dawley rats displayed a similar rate of inhibition of the respiration after sequential addition of 3NP (final 3NP concentration= 8 mM, n=6).

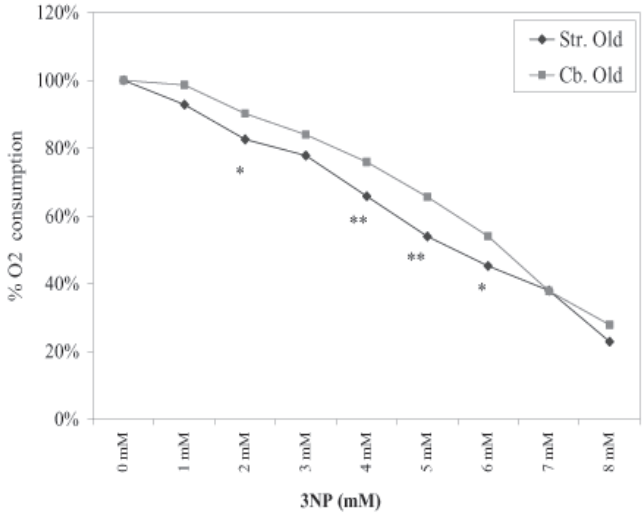

FIGURE 3. Mitochondrial respiration and 3NP treatment in old animals. Similar concentration of 3NP more effectively inhibited mitochondrial respiration in the striatum of the older animals as compared to the cerebellum of the same animals (* $P<0.05$, ** $P<0.01 \pm$ SEM, n=6).

Table 1

The rate of oxygen consumption in state III uncoupled in striatum and cerebellum of young and old rats. Although mitochondrial preparation from younger rats consistently displayed a higher rate of oxygen consumption, upon statistical analysis, no significant difference were observed in oxygen consumption in respect to the age or the region examined.

Mitochondrial Source	State III Respiration(nmol/mg/min)
Striatum-old	37.72 ± 9.69 (n=6)
Cerebellum-old	31.67 ± 6.13 (n=6)
Striatum-young	54.91 ± 11.37 (n=6)
Cerebellum-young	39.29 ± 6.54 (n=6)

ROS Production

In order to investigate whether 3NP application in isolated mitochondria results in production of free radical species, we measured the generation of free radical in mitochondria isolated from the striatum and cerebellum of aged and young rats. In the older animals, 3NP did not increase DCF fluorescence in either striatum or cerebellum. Interestingly, there was a significant reduction of DCF signal in the striatum and cerebellum of younger animals (Fig. 4). The application of 10 μM H_2O_2 as a positive control resulted in increased DCF signal in a linear fashion. As expected, the addition of two concentrations of Ca^{2+} (50 μM and 150 μM) to the isolated mitochondria preparation from the striatum of older animals resulted in a significant concentration-dependent increase in DCF fluorescence signal. Although there was an increase in ROS generation in cerebellar mitochondria in aged animals, the increase was subtler compared to the striatum. The striking finding was the lack of increase in DCF fluorescence in the presence of Ca^{2+} in the striatum and cerebellum of younger animals, since a number of reports have suggested that mitochondria are the principal source for calcium-dependent free radical production (Dugan, Sensi et al. 1995; Reynolds and Hastings 1995; Perez Velazquez, Frantseva et al. 1997). Although previous studies have suggested that Ca^{2+}-mediated changes in mitochondrial function results in pathological stimulation of mitochondrial free radical production (Murphy 1997), our results indicate that Ca^{2+}-mediated free radical production in mitochondria is age-dependent (Fig. 4).

DISCUSSION

The goal of current study was to evaluate the mechanisms underlying the hierarchy of neuronal vulnerability in the 3NP model at the mitochondrial level. We isolated the mitochondria from striatum and cerebellum of two and ten-month old rats. The reason for obtaining the mitochondria from two different age groups was based on previous reports indicating that the older experimental animals display more severe 3NP-induced neurotoxicity (Bossi, Simpson et al. 1993; Brouillet, Jenkins et al. 1993). In the present study, we addressed the ability of 3NP to inhibit the mitochondrial respiration in the striatum and cerebellum of the younger versus the older animals. We also examined the production of ROS in mitochondrial preparations in the presence of 3NP and calcium from the same regions of the brain. We speculated that the rate of

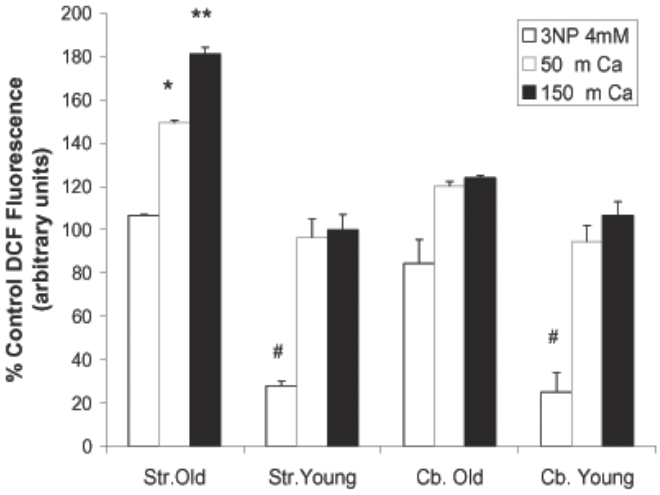

FIGURE 4. ROS production as detected by increased DCF fluorescence signal. 3NP administration (4 mM) in mitochondrial preparation for 15 minutes did not result in a significant change in reactive oxygen species detected by DCF assay, while there was a significant decrease in DCF fluorescence signal in the striatum and cerebellum of the younger animals (# $p<.01$, n=6). The addition of calcium (50 μM and 150 μM) resulted in a significant calcium concentration-dependent increase in ROS production in the striatal mitochondrial preparation of the older animals (* $p<.05$, ** $p<.01$, n=6). However, similar concentrations of calcium did not alter DCF fluorescence signals in either the striatum or cerebellum of younger animals.

ROS generation is likely related to the overall rate of respiration and should differ as a function of age. The primary goal of the current study was to elucidate possible mechanisms that account for selective age-depended striatal vulnerability and increased oxidative stress associated with 3NP model as reported previously (Beal, Ferrante et al. 1995; Schulz, Henshaw et al. 1996; Schulz, Huang et al. 1996; Alexi, Hughes et al. 1998; Alexi, Hughes et al. 1998; Klivenyi, Andreassen et al. 2000; La Fontaine, Geddes et al. 2000; La Fontaine, Geddes et al. 2000). We were also interested in the role of calcium insult in the generation of mitochondrial ROS since the impaired mitochondrial calcium homeostasis is implicated in 3NP-induced toxicity. A number of previous observations have demonstrated that excessive calcium uptake by mitochondria stimulates mitochondrial free radical generation (Dugan, Sensi et al. 1995; Reynolds and Hastings 1995).

Mitochondria are the main source of energy production in the form of ATP via the Krebs cycle and the electron transport chain in mammalian cells. Furthermore, mitochondria are also the primary source of reactive oxygen species, and play a key role in free radical homeostasis in cellular environments. Harman and colleagues first suggested that aging is somehow related to the effectiveness of disposal of oxygen free radicals generated during ATP production (Harman 1981). Examining the respiratory rate of mitochondria from the striatum and cerebellum of younger versus older animals showed no statistical difference between or within different age groups. These data suggest, at least in normal aging, the rate of mitochondrial respiration in the striatum and cerebellum of rats remains the same; however, our data does not indicate whether the efficiency of respiration is retained with age.

The application of 3NP affected the mitochondrial respiration from the striatum and cerebellum of the younger animals at the same rate, while the respiration rate in the striatum of aged animals was more susceptible to 3NP than the cerebellum of the same animals. These results suggest that SDH activity in the striatum and cerebellum respond differently to the aging process. Similar results have been observed in different mammalian tissues. Bowling and colleagues did not observe a decline in SDH activity in brain homogenate of primates (Bowling, Mutisya et al. 1993), whereas Shemyakov and associates reported a decline in SDH activity in hypothalamus of humans (Shemyakov 2001). Other reports have also indicated a different SDH response to aging in different tissues such as cardiac, skeletal and lymphatic tissues in which SDH activity is declined, increased or not changed in aging (Torii, Sugiyama et al. 1992; Sandy, Langston et al. 1993; Drouet, Lauthier et al. 1999). In brief, the cumulative analysis of reports to date indicates that the vulnerability of mitochondrial respiration in aging differs in various organs, tissues and cell populations. Therefore, the variable response of SDH in the striatum and cerebellum of the older animals obtained in our observation is likely due to an intrinsic difference in the striatal SDH as compared to that of the cerebellum in the Sprague-Dawley rodent model.

The addition of 3NP to mitochondrial preparations from the older animals did not result in increased production of ROS in either striatum or cerebellum, while in younger animals 3NP resulted in a significant reduction in ROS generation. The lack of ROS generation after 3NP application can be explained by the unique position of SDH as a member of both the Krebs cycle and the respiratory chain. Irreversible inhibition of SDH by 3NP in the Krebs cycle prevents the conversion of succinate to fumarate; therefore, interrupting the Krebs cycle and reducing the formation of NADH, the main electron carrier in the electron transport chain. The unique position of SDH also interferes with the electron transport chain at complex II by reducing the available $FADH_2$, resulting in suppression of oxidative phosphorylation. Since oxidative phosphorylation is the major source of ROS generation, it is of no surprise that 3NP does not directly increase ROS generation. The fact that ROS generation is attenuated in younger animals could simply be due to more efficient antioxidant properties of mitochondria in younger animals. This interpretation is in agreement with the previous reports indicating that normal aging results in a decline in the efficiency of cellular antioxidant processes (Toescu, Myronova et al. 2000; Toescu and Verkhratsky 2000).

The striking difference in mitochondria ROS generation in response to calcium insult was quite unexpected. The results suggest that mitochondrial calcium increase in the aged rats results in a significant production of ROS, whereas the younger animals are not as susceptible to calcium insults, and no increase in ROS generation is observed in mitochondria from either the striatum or cerebellum of the younger group. These results suggest that in aging, mitochondria become more susceptible to the generation of reactive oxygen species in conditions, such as excitotoxicity, that cause a concurrent mitochondrial calcium increase. This finding implicates mitochondria as a key cellular target in pathological states, such as ischemia, which are closely associated with increases in extra- and intracellular calcium concentration.

In summary, the present study demonstrates that 3NP inhibits the respiratory chain activity in isolated mitochondrial preparation from the striatum and cerebellum of both young and old animals. However, the rate of mitochondrial respiration inhibition is affected differently in striatum and cerebellum of the older animals. Our results indicate that 3NP is a significantly stronger inhibitor of mitochondrial respiration in the striatum as compared to that of cerebellum of the older animals. We have also demonstrated that 3NP inhibition does not increase the production of ROS

in either the young or the mature animals. Since evidence is accumulating to suggest that oxidative stress plays a critical role in 3NP toxicity, further investigation is required to identify other sources of oxidative stress in 3NP-induced toxicity. However, our results clearly demonstrate that 3NP inhibition of mitochondrial respiration does not directly result in increased ROS in isolated mitochondria.

In this perspective, the above studies provide significant novel information regarding the role of mitochondrial impairment in the different regions of brain in aging and a better understanding of mitochondrial calcium homeostasis and its alteration as a function of age. The current challenge is now to better understand and define the mechanisms involved in mitochondrial and cellular homeostasis in aging, and develop new therapeutic approaches to counteract any limitations associated with these mechanisms in pathological condition as well as in normal aging.

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